CHEST

Official publication of the American College of Chest Physicians

Additional Proteins in BAL Fluid of Metsovites Environmentally Exposed to Asbestos : More Evidence of "Protection" Against Neoplasia?

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Chest 2002;121;273-278
DOI 10.1378/chest.121.1.273

Additional Proteins in BAL Fluid of Metsovites Environmentally Exposed to Asbestos*

More Evidence of "Protection" Against Neoplasia?

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Introduction: **Inhabitants of Metsovo in northwest Greece have been exposed to asbestos from use of a tremolite-containing whitewash ("luto" soil). As a result, they have increased incidence of malignant pleural mesothelioma and pleural calcifications (PCs). However, subjects with calcifications have a much lower incidence of mesothelioma than those without. A previous study of the two groups with BAL revealed higher proportional lymphocytosis among subjects with calcifications. We suggested that BAL lymphocytosis may be somehow correlated with "protection" against neoplasia.**
Methods: **The present report is a study of the liquid phase of BAL in the two groups. BAL specimens of 43 Metsovites (13 subjects with PCs and 30 subjects without PCs) and two control groups were examined. We measured total protein, albumin, IgG, IgA, and interleukin-6. Proteins were analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and two-dimensional electrophoresis and further characterized using an appropriate computer program.**
Results: **The most interesting finding was the presence of two additional protein spots corresponding to the electrophoretic site of Ig heavy chain and C_4 component of complement. The two proteins were present in all Metsovites with PCs but in none without PCs and also in none of the control groups.**
Conclusion: **This study further separates two groups of Metsovites with different reaction to asbestos, possibly as a result of different activation of alveolar macrophages. This difference leads the first group to the formation of PCs, BAL fluid lymphocytosis, and relative "protection" against malignancy, and the second group to no calcifications, no lymphocytosis, but also no protection against malignancy.** ***(CHEST 2002; 121:273–278)***

Key words: asbestos; macrophages; Metsovo; neoplasia; pleural calcifications; two-dimensional electrophoresis

Abbreviations: 2-DE = two-dimensional gel electrophoresis; IL = interleukin; MPM = malignant pleural mesothelioma; MW = molecular weight; PAGE = polyacrylamide gel electrophoresis; PC = pleural calcification; pI = pH value for isoelectric point; SDS = sodium dodecyl sulfate

Inhabitants[1] of the Metsovo area in northwest Greece (population 5,000) have been exposed since childhood to asbestos fom a tremolite-containing whitewash ("luto" soil).[1] As a result, they have very high incidence (47% of adult population) of pleural calcifications (PCs)[2,3] and malignant pleural mesothelioma (MPM) [300 times higher than expected in populations not exposed to asbestos].[4] It is interesting, however, that MPM develops in the Metsovites without PCs.[4–6] We combined this finding with the appearance of higher proportional lymphocytosis in the BAL fluid of subjects with PCs and suggested that BAL lymphocytosis and PCs in Metsovites may be an indication of "protection" against neoplasia.[6] We also claimed that the alveolar macrophage may be responsible for all these phenomena.

In a previous study,[6] we mainly examined the cellular profile of BAL specimens. In the present study, we examine the liquid phase of BAL in order to identify products of inflammatory and immunoactive cells in the lungs of Metsovites, products that could enable us to shed some light on the question of different degrees of protection against neoplasia in these subjects.

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Manuscript received August 3, 2000; revision accepted May 17, 2001.
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Materials and Methods

Study Population

After written consent, 43 Metsovites underwent BAL, 13 Metsovites with PCs shown on chest radiography (Fig 1) and 30 Metsovites without PCs, among them 5 patients with MPM and 5 patients with bronchogenic carcinoma. In the latter 10 patients, BAL was performed after completion of fiberoptic bronchoscopy for diagnosis of thoracic malignancy. The other 33 subjects were volunteers. All were aware of their asbestos exposure, and they were told that the purpose of the study was to further investigate this problem. None had diffuse pulmonary disease or acute infection at the time of and at least 1 month before examination. Complete history and physical examination included questions regarding the use of luto. We obtained BAL specimens from two control groups: (1) five patients with primary Sjögren's syndrome and BAL fluid lymphocytosis from a previous study[7]; and (2) five patients, non-Metsovites, with extensive PCs not related to asbestos exposure but to other causes (Fig 2) [hemothorax (n = 2) and presumably previous tuberculous pleurisy (n = 3)]. The two control groups were used in order to exclude the possibility that any findings observed in Metsovites could be related to the BAL fluid lymphocytosis itself or the PCs and not to the asbestos exposure. Study of the second control group (non-Metsovites with PCs) was limited to protein analysis for detection of additional proteins. The characteristics of Metsovites and control groups are shown in Table 1.

Bronchoscopy and BAL

The methodology of bronchoscopy and evaluation of the cellular constituents of BAL fluid have been previously described.[6,7]

Liquid Phase Analysis

Protein Assays: The amount of total proteins was measured according to the method of Lowry et al[8] and expressed as micrograms per milliliter. Albumin, IgG, and IgA were measured using single radial immunodiffusion (Behring OPUS; Westwood, MA). Interleukin (IL)-6 was measured using enzyme-linked immunosorbent assay (R&D Systems; Minneapolis, MN).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis: One-dimensional gel electrophoresis was carried out in 10%, vertical sodium dodecyl sulfate (SDS)-polyacrylamide gel

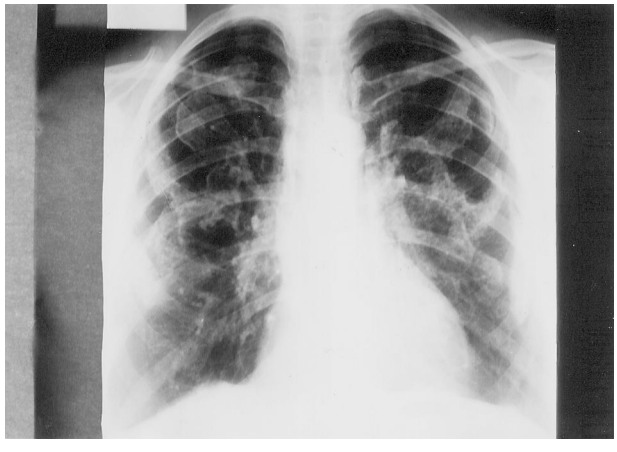

Figure 1. Chest radiograph of a Metsovite with extensive PCs.

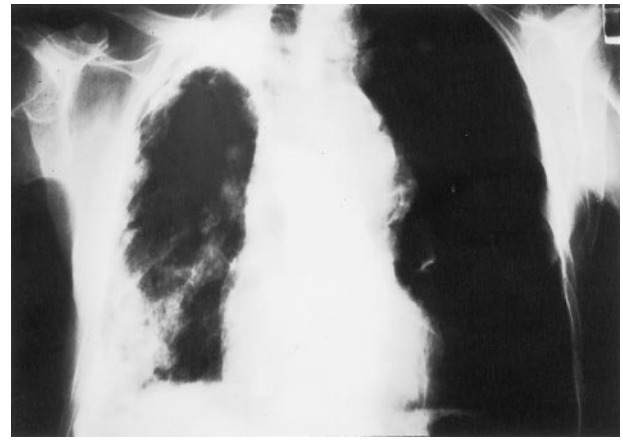

Figure 2. Chest radiograph from a control group patient with PCs from history of tuberculous pleurisy.

(0.14 × 12 × 16 cm). Prior to the gel application, 100 μg protein concentrate was mixed with gel buffer (0.250 mol/L Tris-HCI pH 6.8, 9.2% weight/volume SDS, 40% weight/volume glycerol, 2% weight/volume bromophenol blue, 5% weight/volume mercaptoethanol) and denatured in boiling water for 5 min.[9] High- and low-molecular weight (MW) standards included α_2-macroglobulin (180 kd), β-galactosidase (116 kd), fructose-6 phosphate kinase (84 kd), pyruvate kinase (58 kd), fumarase (48 kd), and carbonic anhydrase (30 kd) [Sigma Chemical; St. Louis, MO].

Two-dimensional Gel Electrophoresis: Two-dimensional gel electrophoresis (2-DE) was performed according to O'Farel[10] with some modifications. Isoelectric focusing in the first dimension was performed with 150 μg of dried samples dissolved in a solubilization mixture containing 9.5 mol/L urea, 10% volume/volume octylphenolpoly(ethylene-glycolether)n, 50 mM dithiothreitol, and 0.8% weight/volume carrier ampholytes (pH 3.5 to 10). Samples were applied anodically to individual isoelectric focusing strips. The gel strips (0.5 × 5 × 110 mm) were prefocused at limited voltage (200 V for 15 min, 300 V for 30 min, and 400 V for 60 min) followed by 18 h at 400 V. Prior to the second-dimensional separation, the gel strips were equilibrated for 20 min at 30°C with gentle shaking in 0.025 mol/L Tris-HCI pH 8.4, 0.192 mol/L glycine, 6 mol/L urea, 30% weight/volume glycerol, 1.6% weight/volume SDS, 1% weight/volume dithiothreitol, and 0.0125% weight/volume bromophenol blue. The second-dimensional (SDS)-polyacrylamide gel electrophoresis (PAGE) [1.4 × 120 × 160 mm] was run in 10% pore-gradient gels.[10] The gels were silver stained by the method of Blum et al.[11] MW standards included $\alpha_2$macroglobulin (180 kd), β-galactosidase (116 kd), fructose-6 phosphate kinase (84 kd), albumin (67 kd), pyruvate kinase (58 kd), ovalbumin (43 kd), lactic dehydrogenase (36 kd), isomerase (26 kd), and trypsin inhibitor (20 kd) [Sigma Chemical].

Analysis Using the Melanie II-2D-PAGE Computer Program: The protein profile of two-dimensional PAGE was compared to reference protein profile using the Melanie II-2D PAGE computer program (Bio-Rad; Richmond, CA), which is designed to analyze images of 2-DE performing qualitative image analysis of 2-DE gels.[12] In brief, all 2-DEs were scanned and stored in the program. Individual points in every gel were marked as landmarks, using the landmark tool. A landmark is defined by its position (row and column number) and its name, and was assigned a pH value for its isoelectric point (pI) and an MW value. The pI and MW values of albumin, α_1-antitrypsin and transferrin were used to compute approximated pI and MW

Table 1—*Population Characteristics and Results of BAL

Patient Groups†	Age, yr	Sex, M/F	Exposure Until, Years Old	Smoking, Y/N	BAL, Cellular Components Cell Populations, %				BAL, Soluble Components				
					Cells, 10^6/mL	MΦ	Lymphocytes	Neutrophils	Total Protein, μg/mL	IgA, mg/dL	IgG, mg/dL	Albumin, mg/dL	IL-6, pg/mL
A	70.5 ± 8.2	6/7	40.1 ± 17.35	5/8	4.2 ± 4.93	65.1 ± 15.89	32.2 ± 14.48	2.6 ± 6.63	110.70 ± 24.37	0.78 ± 0.66	0.95 ± 0.13	5.21 ± 0.60	17.90 ± 11.57
B1	59.8 ± 11.5	11/9	28.6 ± 13.82	10/10	7.2 ± 7.68	83.4 ± 25.43	6.6 ± 4.80	9.4 ± 24.96	78.0 ± 50.71	18.90 ± 57.31	0.88 ± 0.34	4.78 ± 0.61	24.28 ± 8.47
B2	64.4 ± 12.6	1/4	30.7 ± 13.42	2/3	3.3 ± 1.6	81.7 ± 11.14	14.8 ± 11.02	3.4 ± 2.42	109.8 ± 72.42	0.78 ± 0.11	0.80 ± 0.12	4.74 ± 0.47	29.33 ± 2.52
B3	73.8 ± 2.6	5/0	52.3 ± 8.02	1/4	5.5 ± 5.1	85.8 ± 10.03	9.3 ± 8.61	4.4 ± 7.95	79.75 ± 49.68	0.78 ± 0.16	0.69 ± 0.02	4.05 ± 0.66	ND
C1	52.2 ± 9.4	0/6	No	0/6	9.5 ± 7.1	69.2 ± 11.26	30.7 ± 11.26	0	261.20 ± 91.89	1.42 ± 0.77	1.99 ± 0.14	5.92 ± 0.33	28.50 ± 6.14
C2	64.0 ± 5.5	5/0	No	4/1	7.5 ± 4.9	73.1 ± 12.83	15.8 ± 13.66	8.8 ± 7.71	84.8 ± 38.01	ND	ND	ND	ND

*Data are presented as mean ± SD or No. ND = not done; No = no exposure; MΦ = alveolar macrophage.
†Group A = Metsovites with PCs; group B1 = Metsovites without PCs, without neoplasia; group B2 = Metsovites without PCs, with mesothelioma; group B3 = Metsovites without PCs, with bronchogenic carcinoma; group C1 = primary Sjögren's syndrome patients (control group 1); group C2 = patients (no Metsovites) with PCs of unrelated etiology (control group 2).

values for any point on a gel. These landmarks were also used as reference points for operations such as gel alignment and gel matching. The gels were matched to a used-chosen reference gel. The reference gel was Plasma Human me1, MelView. We used all the available features of the program.

Statistical Analysis: The values of the total protein content and the IL-6 determinant were compared among the three groups of patients with the aid of the Kruskal-Wallis statistic. The Mann-Whitney rank sum test was applied for pairwise intragroup comparisons when the Kruskal-Wallis statistic revealed significant differences. The level of significance was set at 95% ($p = 0.05$).

Results

The summary statistics for each group are shown in Table 1. The cellular phase of BAL has been reported in our previous study.[6] In the present study, we analyze only the liquid phase.

Total Protein Content, Albumin, IgA, IgG, and IL-6 Determinants in the BAL Fluid

Our results indicate the following: (1) total protein content differed significantly among the three groups ($p < 0.001$). Metsovites without PCs showed the lowest total protein content, lower than Metsovites with PCs ($p < 0.01$) and Sjögren's syndrome patients ($p < 0.001$; Table 2); (2) there were no statistically significant differences in the albumin, IgG, and IgA contents of BAL fluid between the two groups of Metsovites. All values were higher in Sjögren's syndrome patients (data not shown); (3) IL-6 determinant did not differ significantly among the three groups ($p > 0.05$); however, it was lower in Metsovites with PCs (Table 2).

Protein Analysis of the BAL Fluid Specimens With SDS

There were no differences in the protein profile of the two groups of Metsovites with SDS electrophoresis (Fig 3). Albumin (67 kd) predominates, followed by IgG (150 kd), IgA (180 kd), transferrin (80 kd), and α_1-antitrypsin (50 kd). Patients with primary Sjögren's syndrome had similar protein profiles.

BAL Fluid Specimens Analyzed by 2-DE

The two study groups of Metsovites revealed a similar basic protein profile. However, in the 2-DE of every Metsovite with PCs, two additional protein spots became apparent. The first was identified as having a MW of 55 kd and pI of 7.0 to 7.5, while the second protein spot appeared at a MW of 34 kd and pI 6.2 (X and Y spots in Fig 4, *top*). These protein spots were never observed in any Metsovite without PCs (Fig 4, *bottom*) or in any patient in the two control groups.

Table 2—*Total Protein Content and IL-6 Determinants in BAL Fluid of Metsovites With and Without PCs and Primary Sjögren's Syndrome Patients

Variables	Metsovites With PCs	Metsovites Without PCs	Primary Sjögren's Syndrome
Total protein content, μg/mL	110.70 ± 24.37	58.80 ± 23.61	261.20 ± 91.89
IL-6 determinant, pg/mL	17.90 ± 11.57	24.30 ± 8.47	28.50 ± 6.14

*Data are presented as mean ± SD.

Analysis Using the Melanie II-2D-PAGE Computer Program

The additional protein spots using the Melanie II-2D-PAGE computerized system, and Plasma Human me1, MelView as reference map revealed that these two additional proteins correspond to the heavy chain of Ig (MW, 55 kd; pI, 7.0 to 7.6; spot X) and the C_4 component of complement (MW, 34 kd; pI, 6.2; spot Y).

Discussion

Our previous studies of Metsovites exposed to asbestos since childhood have shown that they can be classified into two distinct groups of different degrees or different types of reaction to the fiber. Individuals of the first group react with PCs and proportional BAL fluid lymphocytosis and appear to be less prone to develop neoplasms.[6] The present study subclassifies further these two groups because only in those with PCs did we detected additional protein spots.

The other BAL fluid constituents showed no differences, except for increased total protein content in Metsovites with PCs and slightly increased levels of IL-6 in Metsovites without PCs (both those with and without neoplasia). IL-6 correlates with neoplasia and autoimmune disorders.[13] Interestingly, our control patients with Sjögren's syndrome had increased IL-6 levels. The fact that the additional proteins were not found in any of the control groups suggests that they are related neither to just BAL lymphocytosis (like that of Sjögren's syndrome patients) nor to just PCs (control group 2). They are related to lymphocytosis and PCs secondary to asbestos exposure only.

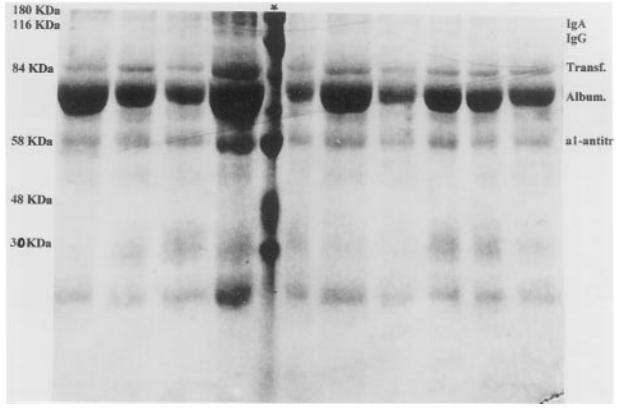

Figure 3. BAL fluid SDS electrophoresis protein profile. The first five columns represent Metsovites with PCs, and the last five columns represent Metsovites without PCs. There are no obvious differences. The position of IgA, IgG, transferrin (Transf.), albumin (Album.), and α_1-antitrypsin (a1-antitr) are indicated. For MW standards, see the "Materials and Methods" section.

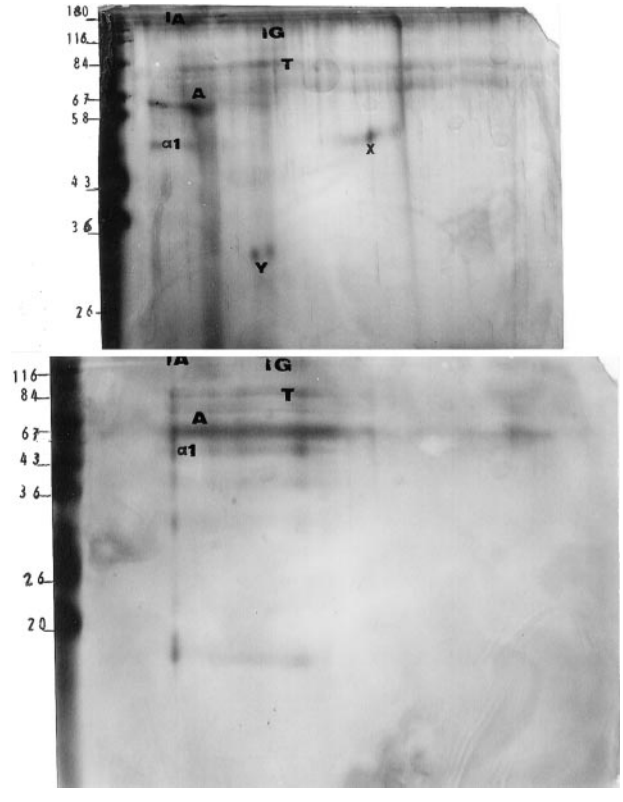

Figure 4. BAL fluid specimens analyzed by 2-DE: (*top*) a Metsovite with PCs and (*bottom*) a Metsovite without PCs. MW standards of the two overlapping ranges were used as indicated in the "Materials and Methods" section. The direction of isoelectric focusing is from left to right (pH, 3.5 to 10). IA = IgA; IG = IgG; T = transferrin; A = albumin; α1 = α_1-antitrypsin. Additional protein spots in a Metsovite with PCs (*top*) are marked with X (55 kd; pI, 7.0 to 7.5) and Y (34 kd; pI, 6.2).

The additional protein spots were only detected using two-dimensional PAGE electrophoresis. This method has been used in the past for detection of proteins present in small amounts in biological fluids, including BAL.[14–16] With the silver stain technique of Blum et al[11] that we used (see the "Materials and Methods" section), amounts of proteins as low as 1 ng can be detected. The specificity of the method is also high, since only one protein with a specific combination of a certain MW and a certain pI occupies a certain position.

The standard protein profile of human BAL fluid in 2-DE was drawn by Lenz et al,[15] and consists mainly of albumin, immunoglobulins (IgA, IgG), transferrin, and α_1-antitrypsin. Our study indicated similar results.

Additional proteins have been previously described in pulmonary diseases. Lenz et al[15] identified a protein spot at a pI of 4.5 and MW of 12 kd only in patients with sarcoidosis. Occupational exposure induces a variety of changes from the standard protein profile in 2-DE.[16] Asbestos-exposed individuals are no exception. Thus, Lindahl et al[17] found three differences in protein spots in BAL fluid specimens from patients with asbestos exposure. The first protein spot (pI, 5.6; MW, 88 kd) was detected in four of five individuals with asbestos pleuritis. The second protein spot (pI, 6.3; MW, 64 kd) was detected in the BAL fluid of the two patients with progressive disease. The third protein spot (pI, 5.0; MW, 48 kd) was increased in patients with pleural plaques. It is difficult to compare our population with these patients mainly because of differences in terminology. The fact remains, however, that both studies identify groups of asbestos-exposed individuals with different protein spots in their BAL fluid.

The additional protein spots found only in Metsovites with PCs were compared with prototype electrophoretic diagrams and were placed at the location of heavy chain of Ig and the C_4 component of complement. Both proteins could be attributed to activated alveolar macrophages.

Alveolar macrophages exposed to asbestos fibers release proteolytic enzymes. Lenz et al[15] attributed the finding of many small-MW proteins seen in BAL fluid of patients with asbestosis to the action of proteolytic enzymes from activated alveolar macrophages. The fragmented Ig in Metsovites with PCs could be due to a similar action of activated alveolar macrophages.

Long-term immunostimulation of alveolar macrophages due to smoking causes increases of IgG, C_3, and C_4.[18] Asbestos inhalation is a similar condition of lung-term immunostimulation and could explain the finding of C_4 in Metsovites with PCs. Certainly, the production of heavy chain Ig and C_4 is not regulated exclusively by alveolar macrophages. Moreover, their activation has many other results not seen in our population. Therefore, the hypothesis of activated alveolar macrophages has to be further confirmed with specific studies.

The results of this study, however, further separate Metsovites into two groups with different types and degrees of reaction to the fiber. In the first group, this reaction leads to PCs, proportional BAL fluid lymphocytosis, additional proteins in BAL fluid, and relative protection against neoplasia. In the second group, this reaction is weaker or different, and there are no PCs, lymphocytosis, and additional proteins, but also no protection against neoplasia.

Could our findings have a potential significance for other populations exposed to asbestos environmentally or occupationally? A large study of such populations could clarify this. If the additional proteins are also found only in those without neoplasia, they could be used to identify susceptible individuals and improve our understanding of asbestos-related neoplasia.

ACKNOWLEDGMENT: The authors thank Dr. Christos Tsapardonis for technical assistance with the Melanie II-2D PAGE program and Mrs. Helen Prevezianou for secretarial assistance.

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